



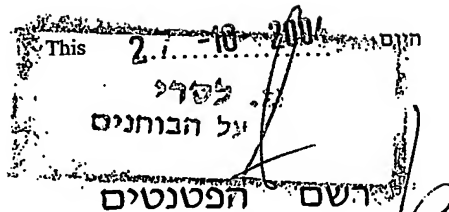
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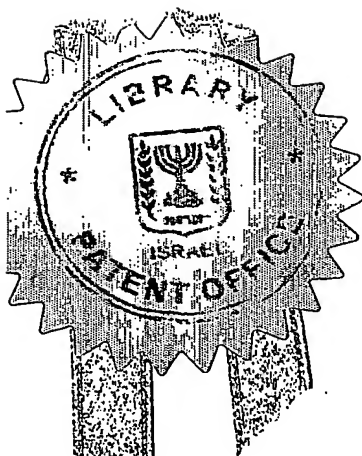
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בקשה לפטנט
Application for Patent

אני, (שם, המבקש, מענו ולגבי גוף מאוגד - מקום התאגדותו)
I (Name and address of applicant, and in case of body corporate - place of incorporation)

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הממציאה: דורית פלאט

owner, by virtue of

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הדין

בעל אמצאה מכח

invention the title of which is

ששמה הוא

(Hebrew)

(בעברית)

תכשירים מיוצבים של פוספטידיל סריין

(English)

(באנגלית)

STABILIZED FORMULATIONS OF PHOSPHATIDYL SERINE

hereby apply for a patent to be granted to me in respect thereof.

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• בקשת חלוקה - Application of Division		• בקשת פטנט מוסף - Application for Patent Addition		דרישת דין קדימה Priority Claim		
מבקשת פטנט from Application		לבקשה/לפטנט to Patent/Appl.		מספר/סימן Number/Mark	תאריך Date	מדינת האגוד Convention Country
No. Dated	מס' מיום	No. Dated	מס' מיום			
• יפוי כח: כללי/פרטי - רצוף/נפרד - עוד יוגש P.O.A.: general/individual - attached/to be filed later הוגש בענין 135466 המערן למסירת מסמכים בישראל Address for Service in Israel LUZZATTO & LUZZATTO P.O. Box 5352 Beer-Sheva 84152 לוצאטו את לוצאטו ת.ד. 5352 באר שבע 84152						
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תכשירים מיוצבים של פוספטידיל סרין

STABILIZED FORMULATIONS OF PHOSPHATIDYL SERINE

Field of the Invention

The invention relates to stabilized phosphatidyl serine preparations and to processes for preparing them. The stabilized phosphatidyl serine preparations of the invention may be in the form of powder, liquid or dispersion. The phosphatidyl serine preparations can be used as nutraceuticals or nutraceutical additives to functional foods or pharmaceutical compositions.

Background of the Invention

Phosphatidyl serine (PS), a phospholipid nutrient, is active in cell membranes and is the major acidic phospholipid component in the membranes of the brain. PS plays a crucial role in many membrane-associated nerve cell processes. The fundamental function of PS is to help maintain proper membrane fluidity, which has major implications on most membrane functions.

PS has been the subject of numerous human clinical trials of memory loss, mood, cognitive performance and learning ability. Many of the studies show that PS can be helpful for those with age-related memory impairment. It can even help to optimize cognition in those with no cognitive impairment.

Dietary PS is efficiently and rapidly absorbed in the intestine, taken up into the blood, and readily crosses the blood-brain barrier to reach the nerve cells of the brain.

PS can be extracted from bovine brain, from plants or it can be produced from soybean lecithin using biocatalysis. By using the transphosphatidylation reaction with phospholipases D (PLDs), the head group of phospholipids can be modified easily. Thus, phosphatidylserine can be produced from phosphatidylcholine or any other phospholipid mixture and serine by catalysis of PLD.

PS is manufactured and marketed in powder and fluid forms, at different concentrations, ranging from 10% to 90%. The fluid form of the PS consists of clear and transparent solution of phosphatidyl serine, usually in oily media of medium-chain triglycerides (MCT) or soy triglycerides.

One of the main difficulties in phosphatidyl serine preparations, especially in liquid form, is its low stability due to rapid decomposition. The high decomposition rates may be due to enzyme traces left in the phosphatidyl serine. If enzyme activity still exists in the PS after encapsulation into soft gel capsules, it will create a new head group exchange, cut off the serine moiety and replace it by glycerol, which is present in the soft gel, whereby phosphatidyl glycerol (PG) is formed.

It is therefore an object of the present invention to provide stabilized PS preparations, as powders, liquids or dispersions.

It is a further object of the present invention to provide methods for the preparation of such stabilized PS preparations.

It is yet a further object of the present invention to provide the said stabilized preparations for use as stand-alone nutraceuticals or as additives to food articles or to pharmaceutical compositions.

These and other objects of the invention will become apparent as the description proceeds.

Summary of the Invention

The present invention relates to a stabilized phosphatidyl serine (PS) preparation, characterized in that said preparation is substantially devoid of phospholipase (PL) activity, particularly phospholipase D (PLD) activity.

The stabilized PS preparation of the invention may be in powder form.

The stabilized PS preparation of the invention may be a liquid preparation comprising PS dissolved in an oil, preferably medium-chain triglyceride. This preparation may further comprise additional functional ingredients, such as lecithin.

The stabilized PS preparation of the invention may be a dispersion of PS in an oil base wherein the PS is substantially devoid of phospholipase activity. The oil base is preferably a triglyceride base, particularly medium-chain triglyceride base or vegetable oil.

The stabilized PS preparations of the invention may be used as nutraceuticals *per se*, and/or as nutraceutical food and/or drug additives. The invention thus further relates to a food article comprising the stabilized PS preparation of the invention. The invention also relates to a pharmaceutical composition comprising the stabilized PS preparation of the invention.

In yet a further aspect, the invention relates to a capsule containing a

stabilized PS preparation according to the invention, which is preferably, but not limited to a soft gelatin capsule.

The stabilized PS preparation of the invention may be used as an enhancer of cognitive performance and learning ability and for preventing memory loss, particularly age-related memory loss.

In a further aspect, the invention relates to a process for the preparation of a stabilized phosphatidyl serine preparation, comprising the steps of:

- a. incubating an aqueous mixture of L-serine with lecithin in the presence of a phospholipase D, preferably Phospholipase D from *Streptomyces sp.* or Phospholipase D from strain of *Actinomadura sp.*, for a suitable period of time to give phosphatidyl serine;
- b. removing and filtering the upper layer which contains the phosphatidyl serine;
- c. washing the filtrate with water to remove excess serine;
- d. washing the resulting phosphatidyl serine with ethanol to remove any traces of phospholipase; and
- e. drying the washed phosphatidyl serine.

In another embodiment the process of the invention may employ a PL which is immobilized on an insoluble matrix and is optionally surfactant coated. In this embodiment, and after step (a), the reaction mixture is allowed to stand until the PLD precipitates.

Still further, the invention relates to a process for preparing a stabilized oil-based liquid preparation of PS comprising the steps of:

- (1) dispersing the PS obtained by said embodiments of the process of the invention in a water:ethanol mixture and stirring at a suitable

temperature;

(2) extracting the PS from the mixture obtained in step (1) with a suitable solvent, preferably n-hexane;

(3) washing the extract obtained in step (2) with water; and

(4) adding an oil, preferably medium-chain triglycerides, to the washed hexane solution obtained in step (3) and evaporating the hexane.

Still further, the invention relates to a process for preparing a stabilized oil-based dispersion of PS comprising the step of dispersing the PS obtained by any of said methods of the invention in a suitable oil base, preferably triglyceride base and particularly medium-chain triglycerides or vegetable oil.

The invention also relates to stabilized PS preparations obtained by the process of the invention.

Detailed Description of the Invention

The present invention relates to stabilized PS preparations, to process for the production of PS preparations and particularly stabilized PS preparations, and to various uses thereof.

The stabilized PS preparations of the present invention are obtained by using specific phospholipases D (PLDs), and by washing the product with ethanol.

Examples of such enzymatic preparations are phospholipase D (Phosphatidyl Choline phosphatidohydrolase, EC 3.1.4.4) from *Streptomyces* sp. or from strains of *Actinomadura* sp. These enzymes are particularly advantageous due to their high reactivity, and yield a highly concentrated PS preparation

(>60%, depending on the raw material).

An additional feature of the process of the invention is the washing of the PS product with ethanol, or other suitable solvent. It is expected that the use of the specific enzymes and the final stage ethanol washings would significantly reduce the decomposition of both power and liquid PS preparations.

In order to obtain yet greater stability of the PS product, the enzyme used in the reaction may be immobilized. The use an immobilized PLD preparation in the process of production of the PS, will prevent enzyme leakage into the final product of the PS, and result in an even more stable PS preparation.

The ethanol washing, optionally with use of a PLD which has been immobilized, yield a product which is substantially devoid of phospholipase activity, and thus less prone to decomposition.

Thus, in a first embodiment, the present invention relates to a high-yield process for preparing a stabilized phosphatidyl serine preparation which is substantially devoid of phospholipase activity, comprising the steps of:

- a. incubating an aqueous mixture of L-serine with lecithin in the presence of a phospholipase D for a suitable period of time to give phosphatidyl serine;
- b. removing and filtering the upper layer which contains the phosphatidyl serine;
- c. washing the filtrate with water to remove excess serine;
- d. washing the resulting phosphatidyl serine with ethanol to remove any traces of phospholipase; and
- e. drying the washed phosphatidyl serine, to give phosphatidyl serine in powder form which is substantially devoid of phospholipase activity.

The phospholipase D is preferably phospholipase D (Phosphatidyl Choline phosphatidohydrolase, EC 3.1.4.4) from *Streptomyces* sp. or from strains of *Actinomadura* sp.

As mentioned, in particular embodiments, the phospholipase may be immobilized on a suitable rigid matrix. The immobilized enzymatic preparation can be filtered off the reaction medium at the end of the reaction. An advantage of this immobilized enzyme preparation is that it can be re-used in many further reaction batches. Matrix-immobilized, preferably surfactant-coated phospholipases can be prepared according to the methods described in WO00/56869, fully incorporated herein by reference.

Thus, in a second embodiment, the invention relates to a process for the preparation of a stabilized phosphatidyl serine preparation which is substantially devoid of phospholipase activity, comprising the steps of:

- a. Incubating an aqueous mixture of L-serine with lecithin in the presence of an immobilized phospholipase for a suitable period of time to give phosphatidyl serine;
- b. allowing the reaction mixture to stand until the phospholipase precipitates;
- c. removing and filtering the upper layer which contains the phosphatidyl serine;
- d. washing the filtrate with water to remove excess serine;
- e. washing the resulting phosphatidyl serine with ethanol to remove any traces of phospholipase; and
- f. drying the washed phosphatidyl serine, to give phosphatidyl serine in powder form which is substantially devoid of phospholipase activity.

In both embodiments, the lecithin is added to an aqueous solution of L-serine, and the mixture is preferably stirred for a suitable period of time, preferably about 1 hour, in order to homogeneously disperse the phospholipids in the aqueous phase.

The enzymatic reaction is carried out for a suitable period of time, preferably at least 24 hours, whilst stirring, and the reaction mixture is then allowed to stand. Where an immobilized enzyme is used, the immobilized enzyme precipitates during this period of time, and can be removed and recycled.

In both embodiments, the upper layer contains the phospholipids dispersion. This dispersion is recovered and filtered, and the resulting phosphatidyl serine is first washed with water, to remove any excess serine, and then washed with ethanol to remove any enzyme residue, and then dried, to give phosphatidyl serine which is substantially devoid of phospholipase activity.

In a further aspect the invention provides stabilized liquid phosphatidyl serine and a process for their preparation.

The process for preparing the stabilized liquid PS preparations comprises the steps of:

- (1) dispersing phosphatidyl serine which is substantially devoid of phospholipase activity, obtained in steps (e) or (f) of the above processes of the invention in a water:ethanol mixture and stirring at a suitable temperature;
- (2) extracting the phosphatidyl serine from the mixture obtained in step (1) with a suitable solvent, preferably n-hexane;
- (3) washing the extract obtained in step (2) with water; and
- (4) adding an oil, preferably, but not limited to medium-chain

triglycerides, to the washed hexane solution obtained in step (3) and evaporating the hexane; to give a stabilized oil-based liquid preparation of phosphatidyl serine which is substantially devoid of phospholipase activity.

In a yet further aspect, the invention provides stabilized dispersions of phosphatidyl serine and a process for their preparation. These are dispersions of phosphatidyl serine in blended oil (e.g., triglyceride-based product such as edible oil, vegetable oil, fish oil, etc.), in which the phosphatidyl serine itself is only dispersed, but is not dissolved in the oily medium.

The process for preparing the stabilized PS dispersions comprises the step of: dispersing the stabilized phosphatidyl serine which is substantially devoid of phospholipase activity, obtained in steps (e) or (f) of the above processes of the invention in a suitable oil base, preferably triglyceride base and particularly medium-chain triglycerides or vegetable oil at a suitable temperature, to give a stabilized oil-based dispersion of phosphatidyl serine is obtained.

This formulation will be checked for the stability of the phosphatidyl serine as an ingredient and in a soft gel capsules.

Another parameter that will be checked is the stability of the oily component in the formulation concerning the peroxide value, and free fatty acids.

It is supposed that due the fact that the phosphatidyl serine is not dissolved in the oil, its stability will increase due to less destructive interactions.

Examples

Enzyme preparations

Materials:

L-Serine: CAS N.56-45-1 (Degussa).

Lecithin: with high concentrations of PC and PE.

Calcium chloride: CALCIOL (Marschall™, Rhodiafood).

Acetic Acid: CAS N 64-19-7 (Acetex Chimie).

Sodium Hydroxide: CAS N. 1310-73-2 (Sigma Chemical Co.)

AEROSIL 200: Manufacture by Degussa.

MCT Crodamol GTCC: Manufacture by Croda.

Titriplex[®] III (ethylenedinitrilotetraacetic acid disodium salt dihydrate)
(Merck KgaA)

Hexane (Sigma-Aldrich).

Enzymes:

1. Phospholipase D from *Streptomyces* sp. (Phosphatidyl Choline phosphatidohydrolase, EC 3.1.4.4), Asahi Chemical Enzymes.
2. Phospholipase D from strain of *Actinomadura* sp., Meito Sangyo.

The main advantage of these enzymes is their high reactivity. Highly concentrated PS is produced (>60%, depending on the raw material). Another advantage of the enzymes is the low concentration (<9%) of phosphatidic acid produced in the process.

Matrix-immobilized, preferably surfactant-coated phospholipase can be prepared according to the methods described in WO00/56869, fully incorporated herein by reference.

Briefly, the crude enzyme (300mg/l protein) is dissolved in 1L tris buffer, pH 6.5 containing 4 gr insoluble inorganic or organic matrix (Celite, silica gel, alumina or polypropylene). The solution is stirred vigorously with a magnetic stirrer for 30 minutes at 25°C. In the case of surfactant-coated immobilized enzyme preparations, sorbitan mono-stearate is added drop-wise to the stirred enzyme solution. All enzyme preparations (i.e. both the surfactant-coated immobilized lipases and the immobilized-crude lipases) are sonicated for 10 minutes and then stirred for 8 hours at 25°C. The formed precipitate is collected by either filtration or centrifugation (12,000 rpm, 4°C), followed by overnight freezing at -20°C and lyophilization.

1. *Preparation of stable phosphatidyl serine in powder form with non-immobilized enzyme*

250 gr of L-Serine were placed in a 1 liter reactor filled with 750 ml phosphate buffer pH 5.6 containing 200mM CaCl₂. After complete dissolution of the serine, 53 gr of fractionated soy lecithin were added.

The mixture was stirred at 40°C for 1 hour, to homogeneously disperse the phospholipid in the aqueous phase.

1.25 gr of enzyme (Phospholipase D from strain of *Actinomadura* sp.) were added to the aqueous dispersion. The reaction mixture was stirred for 24 hours. The upper layer consisting of the phospholipid dispersion was removed from the reactor. The dispersion was filtered and the phosphatidyl serine was washed four times with water to remove the excess serine.

The phosphatidyl serine was washed with ethanol to remove traces of enzyme and dried.

Final weight was 47 gr.

2. *Preparation of stable phosphatidyl serine powdered form using an immobilized enzyme*

250 gr of L-Serine were placed in a 1 liter reactor filled with 750 ml phosphate buffer pH 5.6 containing 200mM CaCl_2 . After complete dissolution of the serine, 53 gr of fractionated soy lecithin were added.

The mixture was stirred at 40°C for 1 hour, to homogeneously disperse the phospholipid in the aqueous phase.

1.25 gr of enzymatic preparation (No. 2 in the following Table 1) were added to the aqueous dispersion. The reaction mixture was stirred for 24 hours and was left without stirring until the enzymatic preparation precipitated at the bottom of the reactor. The upper layer consisting of the phospholipid dispersion was removed from the reactor. The dispersion was filtered and the phosphatidyl serine was washed four times with water to remove the excess serine.

The phosphatidyl serine was washed with ethanol to remove traces of enzyme and dried.

Final weight was 47 gr.

The procedure was repeated with the enzyme preparations shown in the following Table 1. Yields were as indicated for the 1st Batch.

3. *Recycling of the enzymatic preparation*

The enzymatic preparation was re-used in further batches for the production

of phosphatidyl serine as described in Example 2.

Table 1 describes the results obtained with different enzymatic preparations.

Table 1

Enzymatic Preparation	Enzyme	Matrix	Reaction Temperature (°C)	%PS in every batch			
				1 st	2 nd	3 rd	4 th
1	PLD Sangyo	Eupargit 1014F	42	31	61		
2	PLD Assahi	A568	42	39	45	47	43
3	PLD Sangyo	A568	42	46	45	44	55
4	PLD Sangyo	A568	37	39	44	43	44

3. Preparation of stable liquid phosphatidyl serine

The ethanol cake of phosphatidyl serine obtained in Example 1, was dispersed in 0.2M EDTA solution of 1:1 water:ethanol mixture.

The reaction was stirred for 10 hours at 25°C. The phosphatidyl serine was extracted with 250 ml hexane. The hexane layer was washed twice with

water. MCT (95 g) was added to the hexane solution and after evaporation of the hexane, clear oily fluid of phosphatidyl serine was obtained.

It is to be noted that further ingredient may be added to this liquid preparation, in order to enrich the mixture. For example, phosphatidyl choline content may be increased by adding more lecithin.

4. *Preparation of Stable phosphatidyl serine dispersion*

In order to further stabilize the fluid form of phosphatidyl serine, the powdered phosphatidyl serine obtained in Example 1 was dispersed at 25°C in triglyceride base material (for example MCT or Vegetable oil), to give an oil dispersion.

5. *Stability tests*

All the phosphatidyl serine forms including powdered, liquid and the dispersion are checked for their stability as an ingredient in food preparations and as a capsulated product. Using conventional techniques, the phosphatidyl serine concentration is followed and so is the oil stability (for the liquid and the dispersion formulations).

Claims:

1. A stabilized phosphatidyl serine preparation, characterized in that said preparation is substantially devoid of phospholipase activity, particularly phospholipase D activity.
2. The stabilized phosphatidyl serine preparation of claim 1, being in powder form.
3. A stabilized liquid preparation of phosphatidyl serine comprising substantially phospholipase-free phosphatidyl serine dissolved in an oil.
4. The stabilized liquid phosphatidyl serine preparation of claim 4, wherein said oil is medium-chain triglyceride.
5. The stabilized liquid phosphatidyl serine preparation of claim 3 or claim 4, further comprising additional functional ingredients, such as lecithin.
6. A stabilized dispersion of phosphatidyl serine comprising phosphatidyl serine dispersed in an oil base wherein the phosphatidyl serine is substantially devoid of phospholipase activity.
7. The stabilized phosphatidyl serine dispersion of claim 6, wherein said oil base is a triglyceride base, particularly medium-chain triglyceride base or vegetable oil.
8. The stabilized phosphatidyl serine preparation of any one of claims 1 to 7, for use as a nutraceutical food and/or drug additive.

9. A food article comprising the stabilized phosphatidyl serine preparation of any one of claims 1 to 7.
10. A pharmaceutical composition comprising the stabilized phosphatidyl-serine preparation of any one of claims 1 to 7.
11. A capsule containing the stabilized phosphatidyl serine preparation of any one of claims 1 to 7, wherein said capsule is preferably a gelatin capsule.
12. The stabilized phosphatidyl serine preparation of any one of claims 1 to 7, for use as an enhancer of cognitive performance and learning ability.
13. The stabilized phosphatidyl serine preparation of any one of claims 1 to 7, for use in preventing memory loss, particularly age-related memory loss.
14. A process for the preparation of a stabilized phosphatidyl serine preparation, comprising the steps of:
 - a. incubating an aqueous mixture of L-serine with lecithin in the presence of a phospholipase D, preferably Phospholipase D from *Streptomyces* sp. or Phospholipase D from strain of *Actinomadura* sp., for a suitable period of time to give phosphatidyl serine;
 - b. removing and filtering the upper layer which contains the phosphatidyl serine;
 - c. washing the filtrate with water to remove excess serine;
 - d. washing the resulting phosphatidyl serine with ethanol to remove any traces of phospholipase; and
 - e. drying the washed phosphatidyl serine.

15. The process of claim 14, wherein said phospholipase is immobilized on an insoluble matrix and is optionally surfactant coated, and after step (a), the reaction mixture is allowed to stand until the phospholipase D precipitates.

16. A process for preparing a stabilized oil-based liquid preparation of phosphatidyl serine comprising the steps of:

- (1) dispersing the phosphatidyl serine obtained by the process of claim 14 or 15 in a water:ethanol mixture and stirring at a suitable temperature;
- (2) extracting the phosphatidyl serine from the mixture obtained in step (1) with a suitable solvent, preferably n-hexane;
- (3) washing the extract obtained in step (2) with water; and
- (4) adding an oil, preferably medium-chain triglycerides, to the washed hexane solution obtained in step (3) and evaporating the hexane.

17. A process for preparing a stabilized oil-based dispersion of phosphatidyl serine comprising the step of:

- dispersing the phosphatidyl serine obtained by the method of claim 14 or claim 15 in a suitable oil base, preferably triglyceride base and particularly medium-chain triglycerides or vegetable oil.

18. A stabilized phosphatidyl preparation whenever prepared by the process of claim 14 or claim 15.

לוצאטו אה לוצאטו
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